

FORM PTO-1390 OFFICE (REV 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK		ATTORNEY'S DOCKET NUMBER  24615-20146.00	
<b>TRANSMITTAL LETTER TO THE UNITED STATES          DESIGNATED/ELECTED OFFICE (DO/EO/US)          CONCERNING A FILING UNDER 35 U.S.C. § 371</b>				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/869067</b>	
INTERNATIONAL APPLICATION NO.  <b>PCT/NL99/00782</b>		INTERNATIONAL FILING DATE  <b>17/12/99</b>		PRIORITY DATE CLAIMED  <b>22/12/98</b>	
TITLE OF INVENTION <b>PROCESS FOR THE PREPARATION OF <math>\alpha</math>-AMINONITRILES WITH ENHANCED OPTICAL PURITY</b>					
APPLICANT(S) FOR DO/EO/US  <b>Peter J. QUAEDFLIEG, et al.</b>					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (PCT Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> An English language translation of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)). a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). Items 11. to 16. below concern document(s) or information included: 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment. 14. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input type="checkbox"/> Other items or information: *, return receipt postcard.					

**CERTIFICATE OF MAILING BY "EXPRESS MAIL"**

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 K. S. Huff

APPLICATION NO. (if known, see 37 CFR 1.5)

09/869067

INTERNATIONAL

APPLICATION NO. PCT/NL99/00782

ATTORNEY'S DOCKET

NUMBER: 24615-20146.00

CALCULATIONS  
PTO USE ONLY

- 1.
- ☒
- The following fees are submitted:

**BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):**

Neither international preliminary examination fee (37 CFR 1.482)  
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  
and International Search Report not prepared by the EPO or JPO.....\$1,000.00

International preliminary examination fee (37 CFR 1.482) not paid to  
USPTO but International Search Report prepared by the EPO or JPO.....\$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO  
but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO  
but all claims did not satisfy provision of PCT Article 33(1)-(4) .....\$690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO  
and all claims satisfied provisions of PCT Article 33(1)-(4) .....\$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = \$860.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from  
the earliest claimed priority date (37 CFR 1.492(e)).

\$\*

CLAIMS

NUMBER FILED

NUMBER EXTRA

RATE

\$\*

Total claims

21 - 20 =

1

x \$18.00

\$18.00

Independent claims

1 - 3 =

\*

x \$80.00

\$\*

MULTIPLE DEPENDENT CLAIM(S) (if applicable)

+ \$270.00

\$\*

TOTAL OF ABOVE CALCULATIONS = \$878.00

- ☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced  
by 1/2.

\$\*

SUBTOTAL = \$878.00

Processing fee of \$130.00 for furnishing the English translation later than  
☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).

+

\$\*

TOTAL NATIONAL FEE = \$878.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be  
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property

+

\$40.00

TOTAL FEES ENCLOSED = \$918.00

Amount  
to be  
refunded:  
charged: \$\*

- a. ☒ A check in the amount of \$918.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. 03-1952 in the amount of \$\* to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to Deposit Account No. 03-1952. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

for

Kate H. Murashige  
Registration No. (29,959)

09/869067

PATENT

Docket No. 246152014600

531 Rec'd PC

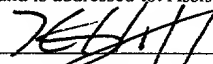
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K.S. Huff

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: U.S. National Phase application of:

Peter J. Quaedflieg, *et al.*

Serial No.: Not yet assigned

Based on PCT Intl'l App: PCT/NL99/00782

Int' Filing Date: December 17, 1999

For: PROCESS FOR THE PREPARATION  
OF  $\alpha$ -AMINONITRILES WITH  
ENHANCED OPTICAL PURITY

Examiner: Not yet assigned

Group Art Unit: Not yet assigned

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

This is a preliminary amendment prior to examination, please amend the claims as follows:

Enclosed is the following Exhibit A:

Exhibit A: Marked-up Version of Amendments to the Claims.

## AMENDMENT

### Please replace presently pending claims 1-12 with the following claims 1-12:

1. (Amended) A process for preparing an  $\alpha$ -aminonitrile with enhanced optical purity which process comprises contacting a mixture of the enantiomers of a chiral N-formyl  $\alpha$ -aminonitrile with an acylase, whereby one of the enantiomers of the N-formyl- $\alpha$ -aminonitrile is selectively deformylated into the unprotected corresponding  $\alpha$ -aminonitrile.
2. (Amended) A process for preparing an  $\alpha$ -aminonitrile with enhanced optical purity which process comprises contacting a mixture of the enantiomers of a chiral (unprotected)  $\alpha$ -aminonitrile with an acylase and a formylating agent whereby one of the enantiomers is selectively converted to the corresponding N-formyl  $\alpha$ -aminonitrile.
3. (Amended) The process of claim 2 wherein the formylating agent is formic acid, a formic acid amide or a formic acid ester.
4. (Amended) The process of claim 1, wherein the acylase is a peptide deformylase having a bivalent metal ion cofactor from group 5-11 of the periodic system.
5. (Amended) The process of claim 1, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.
6. (Amended) The process of claim 1, wherein the peptide deformylase contains the sequences (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.
7. (Amended) The process of claim 4, wherein the peptide deformylase is from *Escherichia coli*.
8. (Amended) The process of claim 4, wherein the bivalent metal is Fe, Ni, Mn or Co.
9. (Amended) The process of claim 8, wherein the bivalent metal is Ni.

10. (Amended) The process of claim 1, which further comprises adding a stabilisation agent.

11. (Amended) The process of claim 10 wherein the stabilisation agent is catalase.

12. (Amended) The process of claim 10 wherein the bivalent metal is Fe.

**Please add the following new claims:**

13. (New) The process of claim 2, wherein the acylase is a peptide deformylase having a bivalent metal ion cofactor from group 5-11 of the periodic system.

14. (New) The process of claim 2, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.

15. (New) The process of claim 2, wherein the peptide deformylase contains the sequences (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

16. (New) The process of claim 13, wherein the peptide deformylase is from *Escherichia coli*.

17. (New) The process of claim 13, wherein the bivalent metal is Fe, Ni, Mn or Co.

18. (New) The process of claim 17, wherein the bivalent metal is Ni.

19. (New) The process of claim 2, which further comprises adding a stabilisation agent.

20. (New) The process of claim 19 wherein the stabilisation agent is catalase.

21. (New) The process of claim 19 wherein the bivalent metal is Fe.

**REMARKS**

The claims have been amended to eliminate multiple claim dependencies and to conform to U.S. practice. The changes to the claims are editorial and do not constitute new matter. Entry of the amendment is respectfully requested.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket No. 246152014600. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: June 19, 2001

By: *for*

*Cy A K Reg No 39183*  
Kate H. Murashige  
Registration No. 29,959

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EXHIBIT A. - VERSION WITH MARKINGS TO SHOW CHANGES MADE

1. (Amended) [Process] A process for [the preparation of] preparing an  $\alpha$ -aminonitrile with enhanced optical purity [wherein] which process comprises contacting a mixture of the enantiomers of a chiral N-formyl  $\alpha$ -aminonitrile [is brought into contact] with an acylase, whereby one of the enantiomers of the [N-formylaminonitrile] N-formyl- $\alpha$ -aminonitrile is selectively deformylated into the unprotected corresponding  $\alpha$ -aminonitrile.
  
2. (Amended) [Process] A process for [the preparation of] preparing an  $\alpha$ -aminonitrile with enhanced optical purity [wherein] which process comprises contacting a mixture of the enantiomers of a chiral (unprotected)  $\alpha$ -aminonitrile [is subjected to a formylation reaction in the presence of] with an acylase and a formylating agent whereby one of the enantiomers is selectively converted [in] to the corresponding N-formyl  $\alpha$ -aminonitrile.
  
3. (Amended) [Process] The process [according to] of claim 2 wherein the formylating agent is formic acid, a formic acid amide or a formic acid ester [is used as a formylating agent].
  
4. (Amended) [Process] The process [according to any] of [claims 1-3] claim 1, wherein the acylase is a peptide deformylase [with] having a bivalent metal ion cofactor [wherein the metal is chosen] from group 5-11 of the periodic system[, is used as acylase].
  
5. (Amended) [Process] The process [according to any] of [claims 1-4] claim 1, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.
  
6. (Amended) [Process] The process [according to any] of [claims 1-5] claim 1, wherein the peptide deformylase contains the sequences [of] (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.
  
7. (Amended) [Process] The process [according to any] of [claims 4-6] claim 4, wherein the peptide deformylase is from *Escherichia coli*.

8. (Amended) [Process] The process [according to any] of [claims 4-7] claim 4, wherein the bivalent metal is [chosen from the group of] Fe, Ni, Mn [and] or Co.

9. (Amended) [Process] The process [according to] of claim 8, wherein the bivalent metal is Ni.

10. (Amended) [Process] The process [according to any] of [claims 1-8] claim 1, [wherein in addition] which further comprises adding a stabilisation agent[ is added].

11. (Amended) [Process] The process [according to] of claim 10 wherein the stabilisation agent is catalase.

12. (Amended) [Process] The process [according to] of claim 10 [or 11] wherein the bivalent metal is Fe.



C L A I M S

1. Process for the preparation of an  $\alpha$ -aminonitrile  
5 with enhanced optical purity wherein a mixture of  
the enantiomers of a chiral N-formyl  $\alpha$ -  
aminonitrile is brought into contact with an  
acylase, whereby one of the enantiomers of the N-  
formylaminonitrile is selectively deformylated  
10 into the unprotected corresponding  $\alpha$ -  
aminonitrile.
2. Process for the preparation of an  $\alpha$ -aminonitrile  
with enhanced optical purity wherein a mixture of  
the enantiomers of a chiral (unprotected)  $\alpha$ -  
15 aminonitrile is subjected to a formylation  
reaction in the presence of an acylase and a  
formylating agent whereby one of the enantiomers  
is selectively converted in N-formyl  $\alpha$ -  
aminonitrile.
- 20 3. Process according to claim 2 wherein formic acid,  
a formic acid amide or a formic acid ester is  
used as a formylating agent.
4. Process according to any of claims 1-3, wherein a  
peptide deformylase with a bivalent metal ion  
25 wherein the metal is chosen from group 5-11 of  
the periodic system, is used as acylase.
5. Process according to any of claims 1-4, wherein  
the peptide deformylase is chosen from the class  
EC 3.5.2.27 or EC 3.5.1.31.
- 30 6. Process according to any of claims 1-5, wherein  
the peptide deformylase contains the sequences of  
(I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.
7. Process according to any of claims 4-6, wherein

the peptide deformylase is from *Escherichia coli*.

8. Process according to any of claims 4-7, wherein the bivalent metal is chosen from the group of Fe, Ni, Mn and Co.
- 5 9. Process according to claim 8, wherein the bivalent metal is Ni.
10. Process according to any of claims 1-8, wherein in addition a stabilisation agent is added.
11. Process according to claim 10 wherein the stabilisation agent is catalase.
- 10 12. Process according to claim 10 or 11 wherein the bivalent metal is Fe.

A B S T R A C T

Process for the preparation of an  $\alpha$ -  
5 aminonitrile with enhanced optical purity wherein a  
mixture of the enantiomers of a chiral N-formyl  $\alpha$ -  
aminonitrile is brought into contact with an acylase,  
whereby one of the enantiomers of the N-  
formylaminonitrile is selectively deformylated into the  
10 unprotected corresponding  $\alpha$ -aminonitrile, and a process  
for the preparation of an  $\alpha$ -aminonitrile with enhanced  
optical purity wherein a mixture of the enantiomers of  
a chiral (unprotected)  $\alpha$ -aminonitrile is subjected to a  
formylation reaction in the presence of an acylase and  
15 a formylating agent whereby one of the enantiomers is  
selectively converted in N-formyl  $\alpha$ -aminonitrile.  
Preferably a peptide deformylase with a bivalent metal  
ion wherein the metal is chosen from group 5-11 of the  
periodic system, is used as acylase, for instance a  
20 peptide deformylase chosen from the class EC 3.5.2.27  
or EC 3.5.1.31. Such peptide deformylases often contain  
the sequences of (I) HEXXH, (ii) EGCLS and (iii)  
GXGXAAXQ.  
The bivalent metal is preferably chosen from the group  
25 of Fe, Ni, Mn and Co, in particular Ni or Fe.

PROCESS FOR THE PREPARATION OF OPTICALLY ACTIVE ALPHA-AMINONITRILES

5

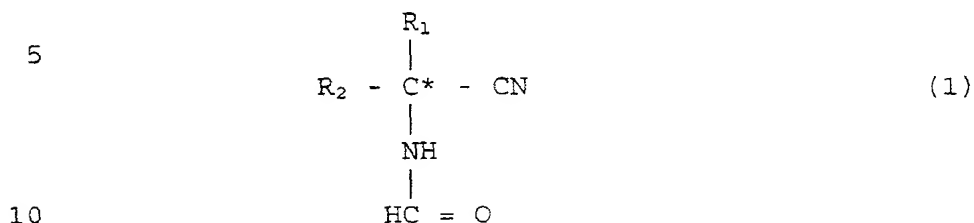
The invention relates to a process for the preparation of an  $\alpha$ -aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of the N-formyl- $\alpha$ -aminonitrile is brought into contact with an acylase, whereby one of the enantiomers of the N-formyl  $\alpha$ -aminonitrile is selectively deformylated into the unprotected corresponding  $\alpha$ -aminonitrile.

There are no processes known in the art wherein a mixture of the enantiomers of an  $\alpha$ -aminonitrile is enzymatically, enantioselectively formylated, or wherein a mixture of the enantiomers of an N-formyl- $\alpha$ -aminonitrile is deformylated.

Applicant now has found that it is possible to remove the N-protecting formyl group enantioselectively from one of the enantiomers of a mixture of the enantiomers of N-formyl- $\alpha$ -aminonitriles. In such enantioselective processes moreover very high E-values can be obtained.

$\alpha$ -Aminonitriles to be used as a substrate in the process of the invention are for instance aliphatic and aromatic  $\alpha$ -aminonitriles, for example the  $\alpha$ -aminonitriles derived from phenylglycine, phenylalanine, *m*-methoxy-phenylalanine, valine and  $\alpha$ -methyl-phenylglycine. In the framework of this invention an  $\alpha$ -aminonitrile is understood to be an  $\alpha$ -aminoacid wherein the carboxy group is replaced by a cyano group.

N-formyl- $\alpha$ -aminonitriles to be used as a substrate are for instance nitriles of formula 1



wherein:

$\text{R}_1$  represent an, optionally substituted, alkyl or aryl group

$\text{R}_2$  represents H, an, optionally substituted, alkyl or aryl group.

The alkyl groups in  $\text{R}_1$  and  $\text{R}_2$  may be cyclic or linear or branched chains. The alkyl and aryl groups may be substituted. Suitable substituents are for instance, hydroxy, alkyl, alkoxy, e.g. methoxy, mercapto, alkylmercapto, amino, guanyl, carboxamide, halogen, e.g. chloro, aryl e.g., phenyl and hydroxy phenyl, imidazolyl or indolyl.

In another embodiment of the present invention a mixture of the enantiomers of a (non-protected)  $\alpha$ -aminonitrile is subjected to a formylation in the presence of an acylase and a formylating agent, whereby one of the enantiomers is selectively converted into the corresponding N-formyl  $\alpha$ -aminonitrile.

Suitable formylating agents are for instance formic acid in case a thermodynamically controlled formylation can be performed, or formic acid esters or amides when the formylation is kinetically controlled. In a thermodynamically controlled formylation the equilibrium is shifted towards the side of the formyl derivative, preferably by precipitation

of the formyl derivative.

Moreover it appeared that, starting from  $\alpha$ -aminonitriles, the non-formylated  $\alpha$ -aminonitriles relatively rapidly racemise at pH values of higher than 5. In such case the optically active N-formyl aminonitrile can be obtained with an enantiomeric excess of more than 90%, in particular more than 95% and with a yield of more than 90%, in particular more than 95%, calculated with respect to the total amount of (racemic)  $\alpha$ -aminonitrile starting product.

Suitable acylases that can be used in the process of the present invention are for instance Penicilline acylases for instance Pen-G or Pen-V acylases, metalloproteases, esterases, deacetylases. Particularly useful enzymes are peptide deformylases.

Peptide deformylases (PDF's) are in general enzymes having formyl methionine peptide deformylase activity. The peptide deformylases to be used according to the invention have a more than 10 times, preferably more than 100 times, in particular more than 1000 times, higher activity towards the N-formyl protected  $\alpha$ -aminonitriles compared to the corresponding N-acetyl protected  $\alpha$ -amino nitriles. Activity here is defined as the catalytic efficiency (also called: specificity constant)  $K_{cat}/K_m$  expressed in  $M^{-1} sec^{-1}$ ; wherein  $K_m$  (expressed in mM) represents the Michaelis constant (this is the substrate concentration at which the reaction rate is 50% of the maximum reaction rate observed) and  $K_{cat}$  (expressed in  $min^{-1}$ ) represents the turnover number. It should be noticed that in the literature also other names are being used instead of the name peptide deformylases; in particular the following names may be mentioned here: formylmethionine

deformylase, N-formylmethionylaminoacyl-tRNA  
deformylase, N-formyl-L-methionine amidohydrolase, N-  
formylmethionyl-aminoacyl-tRNA amidohydrolase.

Suitable peptide deformylases to be used in  
5 the process according to the invention are peptide  
deformylases classified as EC 3.5.1.27. Preferably, the  
enzyme is an enzyme having the activity as described  
for EC 3.5.1.27 because excellent results are being  
achieved in the deformylation with such enzymes. It  
10 should be noticed that until recently it was believed  
that the enzyme coded as EC 3.5.1.31 is catalyzing a  
different reaction. In the meantime, however, it has  
been shown that the enzymes known as EC 3.5.1.27 and EC  
3.5.1.31 are coded for by exactly the same gene and  
15 have the same activity. Therefore, as used herein, the  
term EC 3.5.1.27 is encompassing not only EC 3.5.1.31,  
but likewise all other enzymes having the same activity  
as described for EC 3.5.1.27.

Although the family of PDF's is composed of  
20 proteins with a relatively low level of sequence  
identity, the 3D structures of the members of this  
family appear closely related one to each other with,  
in particular, the building of a common fold around the  
bivalent metal ion and three signature sequences. As is  
25 described (for PDF's indicated as PDF) by Wagner et  
al., J. Biol. Chem., 273, 11413-6 (1998), for many of  
these enzymes characteristically three short amino acid  
stretches are present as strictly conserved motifs,  
namely in that the enzymes contain the sequences (i)  
30 HEXXH, (ii) EGCLS and (iii) GXGXAAXQ. In these  
sequences X represents any natural amino acid, and  
standard one letter codes for amino acids are used: A  
= alanine, C = cysteine, E = glutamic acid, G =  
glycine, H = histidine, L = leucine, S = serine and Q =  
35 glutamine.

Peptide deformylases are obtainable for

instance from eubacteria for example *Escherichia coli*,  
*Bacillus subtilis*, *Clostridium acetobutylicum*,  
*Clostridium beijerinckii*, *Haemophilus influenzae*,  
*Thermotoga maritima*, *Thermus aquaticus*, *Thermus*  
5 *thermophilus*, *Calothrix* PCC 7601, *Bacillus*  
*stearothermophilus*, or *Lactococcus lactis*. Preferably  
an enzym obtainable from *Escherichia coli* is used.

Preferably a peptide deformylase is used  
with a bivalent metal ion whereby the metal is chosen  
10 from the groups 5-11 of the periodic system (New IUPAC  
version; see Handbook of Chemistry and Physics 70th  
edition, CRC Press, 1989-1990, inner page of cover), as  
a cofactor. Preferably the metal is chosen from the  
group of V, Cr, Fe, Ni, Mn, Co, Cu, Pd and Pt, in  
15 particular from the group of Fe, Ni, Mn and Co, most  
preferably Fe or Ni.

Preferably the amount of the bivalent metal  
ions should be about equivalent to the number of moles  
of enzyme. Suitably the molar ratio between these  
20 bivalent metal ions and the number of PDF molecules is  
in the range of 0.6 to 1.4, preferably of 0.8 to 1.2,  
and most preferred the amount of bivalent metal ions is  
equimolar to the enzyme.

Exchange of the bivalent metal ions in the  
25 PDF's in order to obtain PDF enzymes with a co-factor  
as necessary for the present invention can be done by  
the various methods as described in Groche et al.,  
Biochem. Biophys. Res. Comm., 246, 342-346, (1998).  
These methods include simple metal displacement by  
30 incubation of the native enzyme in an excess of the  
desired bivalent metal ion, if necessary preceeded by



the preparation of the apoenzyme via treatment of the native enzyme with a metal chelation compound. Furthermore, the desired bivalent metal ion can already be introduced in (at least part of the enzyme

5 molecules) by using a bacterial growth medium with an enhanced ratio of the desired bivalent metal ion over  $\text{Fe}^{2+}$ .

In addition measures may be taken in order to enhance the stability of the enzyme, for instance

10 the addition of stabilisation agents, for instance catalase, tris-(2-carboxyethyl)phosphine, glucose oxidase, or combinations thereof; or enlarging the concentration of the PDF, for instance to a PDF concentration of at least 0.1 mg of PDF per ml, more

15 preferably of at least 1.0 mg/ml. The upper limit of the concentration of PDF is not critical if practical concentrations are being used. The use of stabilisation measures is especially preferred when an easily oxidisable metal ion, e.g.  $\text{Fe}^{++}$  is present as a cofactor

20 or an easily oxidisable substrate. If not, for instance in case  $\text{Ni}^{++}$  is present as a cofactor, the addition of a stabilisation agent appeared to be superfluous, as the enzyme turned out to be very stable even without stabilisation agent.

25 In addition measures may be taken in order to enhance the stability of the enzyme, for instance the addition of stabilisation agents, for instance catalase, tris-(2-carboxyethyl)phosphine, glucose oxidase, or combinations thereof; or enlarging the

30 concentration of the PDF, for instance to a PDF concentration of at least 0.1 mg of PDF per ml, more preferably of least 1.0 mg/ml. The upper limit of the

concentration of PDF is not critical if practical concentrations are being used. The use of stabilisation measures is especially preferred when an easily oxidisable metal ion, e.g.  $\text{Fe}^{++}$  is present as a cofactor or an easily oxidisable substrate. If not, for instance in case  $\text{Ni}^{++}$  is present as a cofactor the addition of a stabilisation agent appeared to be superfluous, as the enzyme turned out to be very stable even without stabilisation agent.

Alternatively, genetically engineered mutants of PDF's may be used which have for instance enhanced activity or enantioselectivity in the (de)formylation reaction. These mutants can be generated by a number of different approaches; for instance, by site-directed mutagenesis, site-specific random mutagenesis, regio-specific random mutagenesis, and completely random mutagenesis; the latter form of mutagenesis is better known as directed evolution. General applicable methods to perform these different protein engineering approaches are well known to the skilled man. If a random approach will be applied, the mutagenesis cycle will need to be followed by selection of resistant and active mutant(s), thereby leading to the identification of suitable mutants. To obtain PDF mutants also a combination of different protein engineering approaches and/or several rounds of random mutagenesis may be used.

The reaction conditions for the enzymatic deformylation or formylation according to the invention are not very critical and may depend on the substrates

used. Any suitable solvent system which is inert towards the PDF may be applied; such solvents include aqueous systems (solutions or slurries) or aqueous systems also containing a water-miscible organic solvent which is inert under the reaction conditions. Aqueous systems, however, are preferred. Also the concentration of the *N*-formyl compound is not critical, and may be for instance in the range of about 0.1 to 1000 mM. It is not necessary that all of the *N*-formyl compound is dissolved; part of it may be present as a slurry. The concentration of the PDF likewise is not very critical, and usually will be at 0.001 to 100 % by weight of the formyl compound, e.g. at about 0.2 mM of PDF. The pH for the reaction preferably is chosen in the range of 4.0 to 11.0, more preferably of 5.0 to 10.0. The optimum pH is determined by the stability of the  $\alpha$ -aminonitrile and/or the *N*-formyl- $\alpha$ -aminonitrile, and/or the stability and/or activity of the enzyme. The person skilled in the art can easily determine the optimum pH-value. The temperature is not very critical, and suitably will be in the range of 10 to 50°C, e.g. at about 37°C, but for thermostable PDF enzymes higher temperatures may be applied.

In those cases wherein the absolute configuration of the (de)formylated enantiomer was determined, it appeared that the *S*-enantiomer was (de)formylated more rapidly than the *R*-enantiomer. The optical purity is given by the enantiomeric excess (ee), the enantioselectivity of the enzyme is represented by *E*, and calculated as  $k_f/k_s$ , wherein  $k_f$  is

defined as the rate constant of (de)formylation of the most rapidly (de)formylated enantiomer and  $k_s$  is defined as the rate constant of (de)formylation of the least rapidly (de)formylated enantiomer.

5            Optionally a salt promoting hydrophobic interactions is added to the reaction mixture, for instance a sulphate, phosphate, sulphite or acetate of ammonium, Rb, K, Na, Cs or Li. Most preferably ammonium sulphate or lithium sulphate is used.

10

The invention will further be elucidated by the following 3 examples, without being limited thereto.

15    Abbreviations:

TB medium: 12 g/l of Bacto-Tryptone, Difco; 24 g/l of yeast extract, Difco; 4 g/l of glycerole; 2.3 g/l of  $\text{KH}_2\text{PO}_4$ ; 12.5 g/l of  $\text{K}_2\text{HPO}_4$ ;

Hepes: N-2-hydroxyethylpiperazine-N'-2-ethane sulphuric acid;

20

AEBSF: 2-aminoethyl-p-benzene sulphonyl fluoride;

TCEP: tris-(2-carboxyethyl)-phosphine.

MOPS: 3-(N-morpholino) propane sulphonic acid

MES: 2-(N-morpholino) ethane sulphonic acid

25

#### Isolation of PDF(Fe)

For a detailed discussion of the methods used  
30 reference is made to Groche et al., BBRC 246, 342-346 (1998).

PDF(Fe) was isolated from overproducing *E.coli* cells grown at 30°C in 1.6 l TB medium for 14-16 h. About 13 g (wet weight) cell paste were suspended in 26 ml buffer (20 mM Hepes/KOH, 100 mM KF, pH 7.7

5 supplemented with 10 µg/ml catalase from bovine liver (Boehringer Mannheim) and 1 mM AEBSF, disintegrated by sonication (Branson B12, 20 min) at 0°C and centrifuged at 200.000g for 1 h. The clear supernatant (1.3 g of protein; according to biurete reaction) was mixed with

10 1.3 ml 10%(w/v) Polymix G-35 (BASF) adjusted to pH 7.7 and centrifuged at 40.000g for 10 min. The supernatant was applied to a 20 ml Met-Lys-Sepharose column that had been equilibrated with 20 mM Hepes/KOH, 100 mM KF, 0.2 mM TCEP, pH 7.7. After washing with 120 ml of 20 mM

15 Hepes/KOH, 100 mM KF, 0.2 mM TCEP, pH 7.7, PDF(Fe) was eluted with 150 ml 20 mM Hepes/KOH, 100 mM KCl, 0.2 mM TCEP, pH 7.7. The protein containing fractions were concentrated by ultrafiltration using an Amicon PM10 membrane (yield: 140 mg protein, 1400 U/mg; determined

20 according to Groche et al). After adjustment of the TCEP concentration to 1 mM and protein concentration to 40 mg/ml, the PDF(Fe) stock solution (40 mg/ml = 2 mM) was stored frozen at -60°C.

After thawing, the PDF(Fe) stock solution could be used

25 directly in the deformylation experiments described below. If however solutions with lower PDF(Fe) concentrations were required for these deformylation experiments, the PDF stock solution was diluted further in 20 mM Hepes/KOH, pH 7.7, 100 mM KCl, 1 mg/ml bovine

30 serum albumin, 10 µg/ml catalase solution.

### HPLC-analysis

In all cases HPLC conditions had to be developed in which the two deformedylated isomers were separated from each other and from the formylated isomers. To this end two different techniques were applied, i.e. method A and method B, as described below.

From the concentrations of deformedylated isomers in the samples after various reaction times, the (de)formylation rate constant ( $k_f$  and  $k_s$  in  $M^{-1}s^{-1}$ ) could be calculated for both enantiomers, as well as the respective ee values of the deformedylated product. The enantioselectivity of the enzyme (E value) was calculated by taking the ratio  $k_f/k_s$  and is given, as well as the maximum ee value of the deformedylated product observed during the experiments, in the examples below.

#### Method A (without derivatization)

A Crownpak CR(+) column (4x150 mm) was used. Samples (5  $\mu$ l) withdrawn from the deformylation mixture were mixed with 95  $\mu$ l aqueous  $HClO_4$  (10 mM) to inactivate PDF( $Fe^{2+}$ ). Following a brief centrifugation, 20  $\mu$ l of the supernatant were applied to the Crownpak CR(+) column. For specific chromatographic conditions and retention times see the examples II and III.

#### Method B (Precolumn derivatization with

o-Phthaldialdehyde (OPA) and N-acetyl-L-cysteine;

(NAC))

Samples (25 µl) withdrawn from the deformylation mixture were mixed with 25 µl aqueous HClO<sub>4</sub> (100 mM) to inactivate PDF (Fe<sup>2+</sup>). Following a brief centrifugation, 40 µl of the supernatant were added to 80 µl 1 M aqueous H<sub>3</sub>BO<sub>3</sub>/NaOH pH 11, and subsequently 20 µl OPA reagent (consisting of OPA in H<sub>2</sub>O/CH<sub>3</sub>OH 1:1 v/v with a concentration as indicated in the example) was added, and 10 minutes later 20 µl NAC reagent (consisting of NAC in H<sub>2</sub>O/CH<sub>3</sub>OH 1:1 v/v with a concentration as indicated in the example) was added. After 5 min derivatization was terminated by addition of 80 µl (500 mM) aqueous H<sub>3</sub>PO<sub>4</sub>, and 20 µl of the solution were instantaneously applied to a Nucleosil 120-5 C<sub>18</sub> column (250 x 4 mm). Temperature is ambient and detection is spectrophotometric using a wavelength of 257 nm and/or 340 nm; the used eluent is a mixture of 80 mol% aqueous 0.05 M H<sub>3</sub>PO<sub>4</sub> (brought at pH = 7.0 with 1 M NaOH) and 20 vol% CH<sub>3</sub>CN.

Example I: Deformylation of N-formyl-valine aminonitrile in the presence of Li<sub>2</sub>SO<sub>4</sub> at pH = 7.2.

The deformylation reaction of N-formyl-valine aminonitrile was performed in a 1.5 ml Eppendorf reaction test tube. The reaction mixture with a total volume of 200 µl contained 100 mM aqueous MOPS/NaOH, 2 M Li<sub>2</sub>SO<sub>4</sub> buffer pH 7.2, and 10 mM of N-formyl-valine aminonitrile. After thermal equilibration to 37°C the deformylation reaction was started by the addition of

50  $\mu\text{M}$  of PDF. At various reaction times samples of the reaction mixture were withdrawn in which the reaction was stopped by addition of  $\text{HClO}_4$ .

HPLC-analysis was performed according to method B, with

5 [OPA] = 16 mg/ml, and [NAC] = 4 mg/ml, retention times: 8.6 min (L-enantiomeer), 10.2 min (D-enantiomeer).

Results:

10  $E = 47.9$

$ee_{\text{max}} = 95.5$

$k_s = 0.62 \text{ M}^{-1}\text{s}^{-1}$

$k_f = 29.7 \text{ M}^{-1}\text{s}^{-1}$

15 Example II: Deformylation of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile without  $\text{Li}_2\text{SO}_4$  at pH 7.2

The deformylation reaction of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile was performed as described in example I, with the exception that 100 mM  
20 MOPS/NaOH, 250 mM NaCl, 0.1 mg/ml catalase buffer pH 7.2 was used instead of 100 mM MOPS/NaOH, 2 M  $\text{Li}_2\text{SO}_4$  buffer pH 7.2. Furthermore, 7.2 mM of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile and 2.5  $\mu\text{M}$  of PDF were used.

25

HPLC-analysis was performed according to method A

Eluent: 90 vol% 10 mM aqueous  $\text{HClO}_4$ /10 vol%  $\text{CH}_3\text{OH}$

Flow rate: 0.8 ml/min, temperature: 5° C, detection: 210 nm,

30 retention times:



Deformylated enantiomer(s): 23.8 min.

30.7 min.

N-formyl aminonitrile: 52.0 min.

5 Results:

E = 685

$ee_{\max} = 99.0$

$k_f = 1370 \text{ M}^{-1}\text{s}^{-1}$

$k_s = 2 \text{ M}^{-1}\text{s}^{-1}$

10

Example III: Deformylation of N-formyl-phenylalanine  
aminonitrile without  $\text{Li}_2\text{SO}_4$  addition at pH 6.2.

The deformylation reaction of N-formyl-phenylalanine aminonitrile was performed as described  
15 in example I, with the exception that 100 mM MES/NaOH buffer pH 6.2 was used instead of 100 mM MOPS/NaOH, 2 M  $\text{Li}_2\text{SO}_4$  buffer pH 7.2. Furthermore, 7.5 mM of N-formyl-phenylalanine aminonitrile and 20  $\mu\text{M}$  of PDF were used.

20 HPLC-analysis was performed according to method A  
Eluent: 90 vol% 10 mM aqueous  $\text{HClO}_4$ /10 vol%  $\text{CH}_3\text{OH}$   
Flow rate: 0.8 ml/min, temperature: 5° C, detection: 210 nm,  
retention time:

25 deformylated aminonitrile: 11.8 min

15.1 min

N-formyl aminonitrile: 28.6 min.

Results:

30 E = 880

$$ee_{\max} = 98.8$$

$$k_f = 880$$

$$k_s = 1$$

C L A I M S

1. Process for the preparation of an  $\alpha$ -aminonitrile  
5 with enhanced optical purity wherein a mixture of the enantiomers of a chiral N-formyl  $\alpha$ -aminonitrile is brought into contact with an acylase, whereby one of the enantiomers of the N-formylaminonitrile is selectively deformylated  
10 into the unprotected corresponding  $\alpha$ -aminonitrile.
2. Process for the preparation of an  $\alpha$ -aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral (unprotected)  $\alpha$ -  
15 aminonitrile is subjected to a formylation reaction in the presence of an acylase and a formylating agent whereby one of the enantiomers is selectively converted in N-formyl  $\alpha$ -aminonitrile.
- 20 3. Process according to claim 2 wherein formic acid, a formic acid amide or a formic acid ester is used as a formylating agent.
4. Process according to any of claims 1-3, wherein a peptide deformylase with a bivalent metal ion  
25 wherein the metal is chosen from group 5-11 of the periodic system, is used as acylase.
5. Process according to any of claims 1-4, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.
- 30 6. Process according to any of claims 1-5, wherein the peptide deformylase contains the sequences of (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.
7. Process according to any of claims 4-6, wherein

the peptide deformylase is from *Escherichia coli*.

8. Process according to any of claims 4-7, wherein the bivalent metal is chosen from the group of Fe, Ni, Mn and Co.
- 5 9. Process according to claim 8, wherein the bivalent metal is Ni.
10. Process according to any of claims 1-8, wherein in addition a stabilisation agent is added.
11. Process according to claim 10 wherein the  
10 stabilisation agent is catalase.
12. Process according to claim 10 or 11 wherein the bivalent metal is Fe.

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PROCESS FOR THE PREPARATION OF  $\alpha$ -AMINONITRILES WITH ENHANCED OPTICAL PURITY .  
 the specification of which is attached hereto unless the following box is checked:

☒ was filed on 17 December 1999 as PCT International Application No. PCT/NL99/00782.

(I~) hereby state that I have reviewed and understand the contents of the above-mentioned specification, including the claims, as amended by any amendment referred to above.

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98204370.5	Europe	22 December 1998	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No

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Application Serial No.	Filing Date	Status
PCT/NL99/00782	17 December 1999	<input type="checkbox"/> Patented <input checked="" type="checkbox"/> Pending <input type="checkbox"/> Abandoned

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